

Use of Polymerase Chain Reaction for Diagnosis of Picornavirus Infection in Subjects with and without Respiratory Symptoms

S. L. JOHNSTON,^{1*} G. SANDERSON,¹ P. K. PATTEMORE,^{1†} S. SMITH,¹ P. G. BARDIN,¹
C. B. BRUCE,^{2‡} P. R. LAMBDEN,³ D. A. J. TYRRELL,^{2‡} AND S. T. HOLGATE¹

Immunopharmacology Group¹ and Department of Microbiology,³ University of Southampton, Southampton General Hospital, Southampton SO9 4XY, and MRC Common Cold Unit, Salisbury,² United Kingdom

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Rhinoviruses and enteroviruses are the major members of the picornavirus genus that cause human disease. We compared the polymerase chain reaction and viral culture for the identification of picornaviruses in nasal aspirates from children during episodes of respiratory symptoms and when asymptomatic and from asymptomatic adults. One hundred eight children, aged 9 to 11 years, completed a year-long study. Within 24 to 48 h of a report of respiratory symptoms, a nasal aspirate was taken in the home. Nasal aspirates were also taken from 65 of the children and from 33 normal adults when they had been free of respiratory symptoms for at least 2 weeks. Picornaviruses were isolated by culture for three passages in Ohio HeLa cells in rolling tubes at 33°C and pH 7.0. For the polymerase chain reaction, duplicate 50- μ l samples were amplified with conserved primers from the 5' noncoding region. Picornaviruses generated ~380-bp bands in agarose gel electrophoresis; the specificity of these bands was confirmed by filter hybridization with a conserved internal probe. Picornaviruses were isolated by culture in 47 (46 rhinoviruses) of 292 symptomatic episodes (16%), whereas the polymerase chain reaction identified picornavirus genomic material in 146 episodes (50%), including all but one of the culture-positive episodes. As for asymptomatic samples, eight (12%) children and two (4%) adults were positive by the polymerase chain reaction, whereas only one child's specimen was positive by culture. This polymerase chain reaction assay represents a clear advance in the identification of picornavirus infection, with a detection rate threefold greater than the virus culture method.

Human rhinoviruses (HRV) and enteroviruses (EV) are the clinically important human pathogens in the *Picornavirus* genus. HRV is the major cause of the common cold and is increasingly recognized as a cause of more serious lower respiratory tract disease (14, 15) as well as exacerbations of asthma (17). The recent identification of the major group HRV receptor as intercellular adhesion molecule 1 (ICAM-1) (7) has improved the prospects for the development of effective anti-HRV therapy (4, 18). Rapid, accurate diagnosis is essential for effective prophylactic or acute treatment strategies and is desirable for prognostic and epidemiologic purposes.

Conventional diagnostic methods for HRV are limited by the poor sensitivity of cell cultures, and serologic diagnosis is virtually impossible because of the large number of serotypes and limited cross-reactivity. The efficacy of diagnosis of HRV with cDNA probes from the 5' and 3' noncoding regions is disappointing (5), but the use of oligonucleotide probes complementary to conserved regions within the 5' noncoding region permitted identification of HRV infections in nasal washings from experimentally induced colds (2). However, when we applied the same oligonucleotide probes to nasal aspirate (NA) samples, unacceptable levels of nonspecific binding were found (unpublished observations).

The polymerase chain reaction (PCR) has recently been

used for the identification of both HRV (6, 12, 16) and EV (3, 12, 16, 19), although the majority of these studies did not examine clinical samples, and those that did studied very small numbers and found PCR to be at best equivalent to virus isolation (16).

We have compared three different pairs of picornavirus-specific primers (two that distinguish between HRV and EV on the basis of the size of the PCR product and one that does not) and have compared the best PCR method with the results of virus isolation for a total of 410 NA samples taken from children with and without and adults without signs and symptoms of viral respiratory tract infection.

MATERIALS AND METHODS

Subjects. As part of a longitudinal study of the role of virus infections in children with respiratory symptoms, 114 children aged 9 to 11 years who had reported wheezing and/or a persistent cough in response to a postal questionnaire and lived in Southampton, United Kingdom, or the surrounding community recorded upper and lower respiratory symptoms daily and peak expiratory flow rate twice daily on a diary card, for 13 months from 3 April 1989 to 30 April 1990. Children recorded a range of upper and lower respiratory tract symptoms, and parents were asked to telephone an investigator immediately if an episode of either upper or lower respiratory symptoms occurred, if the peak expiratory flow rate fell by >50 liters/min from the normal value for that child, or if the parent believed that the child was developing a cold. In such an event (an episode), the child was visited at home within at most 48 h, and in most cases within 24 h, by

* Corresponding author.

† Present address: Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

‡ Present address: PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, United Kingdom.

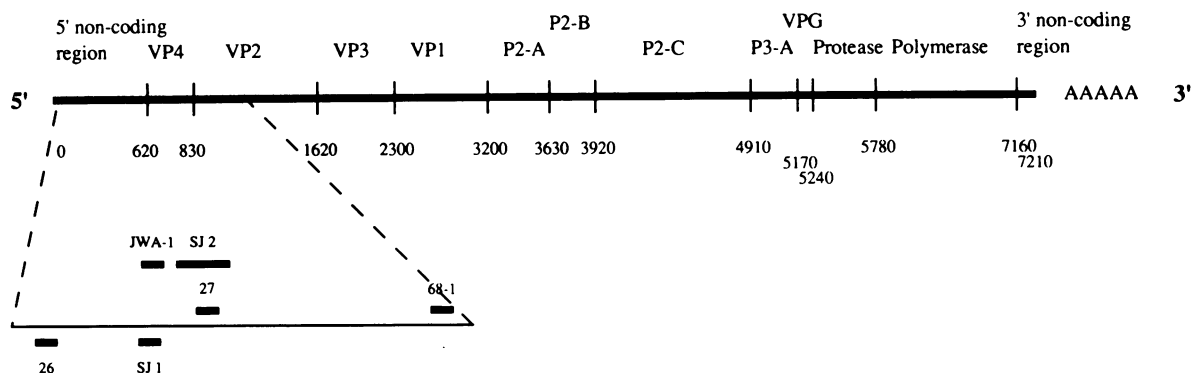


FIG. 1. Diagrammatic representation of the genome of a typical picornavirus. The relative positions of the primers and probes used are indicated in the expanded section. Initiation and cleavage sites are indicated by vertical bars; their approximate positions (below the map) and the relevant proteins coded for (above the map) are also indicated.

one of the investigators to obtain specimens for virologic testing.

The study was approved by the Southampton Hospitals Joint Ethical Subcommittee.

Clinical specimens. (i) **Episode samples.** At each episode, an NA sample was taken by using a sterile mucus trap (Vygon UK Ltd., Gloucester, United Kingdom) connected to a portable foot pump. An aliquot of 10 ml of virus transport medium (10% Hanks balanced salt solution [Flow Laboratories, Irvine, United Kingdom], 0.5% bovine serum albumin, 0.015 M sodium bicarbonate, 20 µg of amphotericin per ml, 40 µg of ciprofloxacin per ml) was added to the extracted mucus, and the mucus and medium were mixed by repeated syringing through a quill. The sample was then aliquoted into sterile microcentrifuge tubes which were immediately sealed, frozen in dry ice, and transported back to the laboratory for storage at -70°C .

(ii) **Control samples.** NA samples were taken from 65 of the same children 15 months after completion of the longitudinal study, when they had been well for at least 2 weeks, and 53 asymptomatic samples were obtained from 35 healthy adults aged less than 65 years. Rhinorrhea was induced by spraying into each nostril 0.1 ml of a solution of histamine (BDH Chemicals, Poole, United Kingdom) made up freshly each day in 0.9% sodium chloride to a concentration of 8 mg/ml. Sampling and storage methods were otherwise identical to those described above.

Picornavirus culture. The picornavirus culture methods used in this study were those in use at the MRC Common Cold Unit (1). Briefly, NA samples were thawed at 37°C , and 0.2 ml was inoculated onto monolayers of Ohio HeLa cells in tubes. After adsorption for 1 h, the samples were decanted and replaced with fresh maintenance medium. The culture tubes were continuously rolled at 5 rpm at 33°C and examined for cytopathic effect (CPE) on days 1, 4, and 7 after inoculation. All cultures were harvested on the seventh day and after freeze-thawing and clarification were subjected to a second passage. For samples where CPE was not clear-cut after the second passage, a third passage was undertaken. The identity of an isolate was confirmed by CPE and by acid stability testing (8).

VRC. Previous workers have emphasized the necessity of the presence of an RNase inhibitor and have advocated the use of vanadyl ribonucleoside complexes (VRC) at a final concentration of 10 mM (6, 16). We elected to use VRC at a final concentration of 20 mM, as the samples in this study

contained more concentrated nasal mucus, and therefore more RNase, than the nasal washes (6) or nasal swabs (16) used in the previous studies. Although we tried many different methods of nucleic acid extraction, samples containing VRC failed to produce consistent results because of carryover of VRC (data not shown). Samples stored without the addition of VRC were therefore used for the rest of the study.

Sample processing for PCR. Duplicate 50-µl aliquots were taken from two different stored microcentrifuge tubes and run on different days to minimize the risk of carryover contamination. Samples were run in batches of 38 comprising 32 samples from children's symptomatic episodes, 4 children's control samples, 1 negative control sample (TE buffer [10 mM Tris, pH 7.4, 1 mM EDTA]), and 1 positive control sample (TE buffer with 5×10^4 50% tissue culture infective doses [TCID₅₀] of HRV 14). Duplicate samples of all children's and adult control specimens were also run on separate occasions in batches of 36 samples with buffer controls only.

Extraction of nucleic acids. Aliquots of sample (50 µl) were subjected to proteinase K (0.4 mg/ml) digestion for 60 min and then subjected to a single phenol-chloroform-isoamyl alcohol (50:50:2) extraction and ethanol precipitation.

Primers. Oligonucleotide primers were synthesized on a model 381A automated DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) by using β-cyanoethyl phosphoramidite chemistry. The primers used were OL68-1 (16), OL27 (6), OL26 (6), and OLSJ-1 (5' TCCTCCGGCCCCCT GAATG 3'), which is complementary to the antigenomic-sense RNA at positions 444 to 461 in the 5' noncoding region of HRV 1B (11) (Fig. 1 and 2). The combinations of primers used and the expected PCR product sizes were as follows: (i) OL68-1 and OL26 (product sizes, 900 bp for HRV and 1,020 bp for EV), (ii) OL68-1 and OLSJ-1 (product sizes, 630 bp for HRV and 750 bp for EV), and (iii) OL27 and OL26 (product size, 380 bp for both HRV and EV).

Initial studies comparing the three pairs of primers demonstrated that OL27-OL26 was the best, in terms of both limiting dilution studies and detection rates of positive samples in a subgroup of the clinical samples (see below). OL27 and OL26 were therefore used for analysis of all 410 clinical samples processed for the comparison with virus isolation.

Reverse transcription. Dried nucleic acid pellets were taken up in 10.5 µl of water. To this mixture were added 0.75

DNA synthesis ----->		<----- DNA synthesis	
OL 26	5' GCACCTCTGTTTCCCC 3'	3' CCNACCACCACCTTAAATGG 5'	OL 68-1
HRV 1B	GCACCTCTGTTTCCCC	GGATGGTGGTGGAAATTACC	
HRV 89	
HRV 14	
HRV 2	
Polio 1	
Polio 2	
Polio 3	
CAV 21	
CAV 9	
CBV 1	
CBV 3	
CBV 4	

DNA synthesis ----->		<----- DNA synthesis	
OL SJ-1	5' TCCTCCGGCCCTGAATG 3'	3' CCNACCACCACCTTAAATGG 5'	OL 68-1
HRV 1B	TCCTCCGGCCCTGAATG	GGATGGTGGTGGAAATTACC	
HRV 89	
HRV 14	
HRV 2	
Polio 1	
Polio 2	
Polio 3	
CAV 21	
CAV 9	
CBV 1	
CBV 3	
CBV 4	

DNA synthesis ----->		<----- DNA synthesis	
OL 26	5' GCACCTCTGTTTCCCC 3'	3' GATGAAACCCACAGGC 5'	OL 27
HRV 1B	GCACCTCTGTTTCCCC	CTACTTTGGGTGCCG	
HRV 89	
HRV 14	
HRV 2	
Polio 1	
Polio 2	
Polio 3	
CAV 21	
CAV 9	
CBV 1	
CBV 3	
CBV 4	

FIG. 2. The sequences of primers used for amplification and the complementary sequences of known HRV and EV are shown to illustrate homology. N is any nucleotide (A, G, C, or T), and Y is either T or C. A dot indicates identity with the reference (HRV 1B) sequence. CAV, coxsackievirus A; CBV, coxsackievirus B.

μ l (100 U) of Superscript (Bethesda Research Laboratories, Paisley, United Kingdom), 4 μ l of 5 \times buffer and 2 μ l of 1 mM dithiothreitol (both supplied with the enzyme), deoxynucleoside triphosphates (dNTPs) to a final concentration of 0.5 mM each (Pharmacia, Milton Keynes, United Kingdom), 0.5 μ l of primer OL68-1 or OL27 (400 μ g/ml), 0.5 μ l (20 U) of RNAGuard (Pharmacia), and water to a final volume of 20.25 μ l. The samples were incubated at 37°C for 60 min.

PCR. The PCR was performed on the total volume of the reverse transcription reaction mixture without further purification in a total volume of 50 μ l by the addition of 5 μ l of 10 \times PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 25 mM MgCl₂), dNTPs to a final concentration of 0.2 mM each, 0.5 μ l of each pair of primers (400 μ g/ml), and 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). After the mixture was overlaid with oil, the reaction cycle was carried out 30 times with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 4 min.

Agarose gel electrophoresis. Ten-microliter aliquots of DNA samples from PCRs were run on horizontal, submerged 1.5% agarose gels in TAE (40 mM Tris acetate, 1 mM EDTA [pH 7.7]) at 120 V for 1 h and stained with ethidium bromide.

Internal probe hybridization of PCR products. (i) **Preparation of samples for filter hybridization.** Ten microliters of PCR product was mixed with 10 μ l of a 3:2 mixture of 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–37% formaldehyde and incubated at 65°C for 15 min. The

samples were applied to nitrocellulose membranes (63 by 288 mm; pore size, 0.45 μ m; Schleicher & Schuell, Dassel, Germany) previously soaked in 10 \times SSC with the aid of a slot blot manifold (Minifold II; Schleicher & Schuell). The filters were baked for 2 h at 80°C and were prehybridized individually in glass bottles (Hybaid, Teddington, United Kingdom) containing 20 ml of prehybridization buffer (4 \times SSC, 10 \times Denhardt's reagent, 400 μ g of sonicated herring sperm DNA per ml, 2.5 mg of *Saccharomyces cerevisiae* RNA per ml) at 47°C (JWA-1) or 65°C (OLSJ-2) for 2 h.

(ii) **Oligonucleotide probes.** Oligonucleotide probes were synthesized by the methods described above. Oligonucleotide JWA-1 (2) was used for the detection of OL27-OL26 PCR products. Oligonucleotide SJ-2 (5' AAACACGGACA CCCAAAGTAGTIGGTCCCTCCGIAITTRCKCATTAC GAC 3'), which was used for the detection of OL68-1-OL26 and OL68-1-OLSJ-1 PCR products, was complementary to nucleotides 511 to 562 of HRV 1B (11). The mixed oligonucleotide SJ-2 contains inosine bases at positions 23, 30, 36, and 38 and degeneracies at positions 41 and 43 (R is G or A; K is G or T) (Fig. 3).

Probe hybridization. 5'-end labelling of the probe was performed with T4 polynucleotide kinase and [γ -³²P]ATP. The reaction was carried out in a total volume of 100 μ l containing 10 μ l of 10 \times polynucleotide kinase buffer (0.5 M Tris-HCl [pH 7.6], 0.1 M MgCl₂, 0.01 M dithiothreitol, 0.002 M EDTA), 8 μ l of oligonucleotide (400 μ g/ml), 10 μ l of [γ -³²P]ATP (10 mCi/ml; 5,000 Ci/mmol; Amersham, Amersham, United Kingdom), and 10 μ l of polynucleotide kinase (2 U/ μ l; Bethesda Research Laboratories) at 37°C for 1 h. The labelled oligonucleotides were purified by gel filtration on Sephadex G-25 (Sigma, Poole, United Kingdom). The specific activities of the probes were approximately 4 \times 10⁸ to 6 \times 10⁸ cpm/ μ g. The filters were incubated in the presence of labelled probe (50 μ l in 20 ml of prehybridization buffer) for 15 h at the same temperature as for prehybridization and then washed as follows: twice at the same temperature in 1 \times SSC–0.2% SDS for 5 min each time, then in 0.1 \times SSC–1% SDS for 20 min, and finally in 0.05 \times SSC–2% SDS for 10 min. The filters were then exposed to X-ray film (Kodak XAR-5; Sigma) at room temperature for 30 min.

Southern blot analysis. The PCR products were transferred from the agarose gels to nitrocellulose membranes (0.45- μ m pore size; Schleicher & Schuell) with an LKB Vacugene vacuum blotter. The membranes were then processed as described above.

Reading of PCR results. (i) **Agarose gels.** Only samples with clearly visible bands of the correct size for the primer pairs in both of the duplicate samples were considered to be positive by gel electrophoresis.

(ii) **Probe hybridization.** X-ray films were read without prior knowledge of the culture results or of whether the samples originated from controls or episodes. Any visible signal that was present in both duplicates was taken as positive.

RESULTS

Of the 114 children, 108 completed the study, and 94 children reported a total of 292 episodes. Satisfactory (0.5 to 1.5 ml of mucus) NA samples were obtained from all reported episodes and all control samples were also satisfactory. The 65 children providing control specimens all came from the group of 94 who had reported episodes; thus, 69% of children reporting episodes also provided control samples.

SJ-2	3'	CAGCATTACKCRTT I A I GCCCTI CCCTGGI TGATGAAACCCACAGGCACAA A	5'
HRV 1B		GT CGT AAT GAGCAA T T GCGGGAT GGGAC CGACT ACT T T GGGT GT CCGT GT T T	
HRV 89		
HRV 14		
HRV 2		
Polio 1	 C* T* G* A*	
Polio 2	 C* T* T* G* A*	
Polio 3	 C* T* G* A*	
CAV 21	 T* T* G* A*	
CAV 9	 C* G* T* CAG* A*	
CBV 1	 C* G* T* CAG* A*	
CBV 3	 C* G* T* CAG* A*	
CBV 4	 C* G* T* CAG* A* G*	
JWA-1	3'	GAGGCCGGGACTT A C G	5'
HRV 1B		CTCGGCCCT GAAT GC	
HRV 89	 T	
HRV 14		
HRV 2	 T	
Polio 1		
Polio 2		
Polio 3		
CAV 21		
CAV 9		
CBV 1		
CBV 3		
CBV 4		

FIG. 3. The sequences of internal probes used for hybridization to amplification products and the complementary sequences of known HRV and EV are shown to illustrate homology. R is either G or A, and K is either G or T. A dot indicates identity with the reference (HRV 1B) sequence. CAV, coxsackievirus A; CBV, coxsackievirus B.

Comparison of PCR primer pairings. In a pilot study of control samples containing limiting dilutions of HRV 14, the detection limits of the three pairs of primers were 10^6 , 10^6 , and 10^3 TCID₅₀/ml by gel electrophoresis and 10^6 , 10^5 , and 10 TCID₅₀/ml by internal-probe hybridization for OL68-1-OL26, OL68-1-OLSJ-1, and OL27-OL26, respectively.

The detection rates of the pairs of primers were also examined by testing (i) 24 episode samples, 11 of which were HRV positive by culture, and (ii) 30 of the samples that were all strongly positive by PCR with OL27-OL26 but negative on culture, with all three primer pairings. The pooled results from all 54 samples used for the comparison between the different pairs of primers are presented in Table 1. OL27-OL26 was the best primer pair, having a much higher detection rate than the other two and the lowest rate of culture-positive and PCR-negative samples.

Comparison of PCR with culture. (i) **Cell culture.** Typical HRV CPE was observed in 47 (16%) of the reported episode samples. The virus in 46 of these samples was regarded as HRV, since the agent was acid labile. The remaining agent, although acid stable, had the CPE characteristics of an HRV rather than an EV and has not yet been definitely classified. All of the adult control samples were negative by culture, and only one (1.5%) (designated an HRV on the basis of both

CPE and acid lability) of the children's control samples was positive by culture.

(ii) **PCR.** (a) **PCR, gel electrophoresis, and ethidium bromide staining.** Bands of 380 bp were seen in both duplicates in 119 (41%) of the 292 episode samples; 43 of these were also positive by culture. Gel electrophoresis thus revealed a further 76 (26%) positive samples compared with culture alone but missed 4 of the 47 (1% of the 292 episode samples) samples that were positive by culture (Fig. 4). Four (6%) of the children's control samples and two (4%) of the adult control samples were positive by gel electrophoresis.

(b) **PCR and internal-probe hybridization.** Positive signals were obtained from 146 (50%) of the episode samples, 46 of which had also been positive by culture (Fig. 5). An additional 100 (34%) positive samples compared with culture alone and an additional 27 (9%) positive samples compared with gel electrophoresis were yielded. A single sample (0.5%) which was positive by culture remained negative by probe hybridization (this sample yielded a positive band in gel electrophoresis when amplified by OL68-1-OLSJ-1, suggesting sequence divergence in one of the sites of the two original primers OL27 and OL26). All 119 samples that were positive by gel electrophoresis were also positive by probe hybridization, confirming the picornavirus specificity of the gel bands (Fig. 4). Further confirmation of picornavirus specificity was provided by Southern blot analysis. Each of the first 36 samples that were positive by gel electrophoresis but negative by culture hybridized at 380 bp; similar confirmation was carried out with 36 of the children's control samples (Fig. 6). There was no evidence of contamination throughout the PCR assay, as all of the duplicate samples were consistent in their results. Of the children's control samples, eight (12%) were positive, including the four that had been positive by gel electrophoresis, and only the two adult control samples that were positive by gel electrophoresis were positive by probe hybridization (Fig. 4).

TABLE 1. Results of PCR with gel electrophoresis: comparison between primer pairs in episode samples

Primer pair for PCR	No. of samples:			
	Processed	Culture positive	PCR positive	Culture positive and PCR negative
OL27-OL26	54	11	47	1
OL68-1-OLSJ-1	54	11	16	6
OL68-1-OL26	54	11	5	9

	Total samples	Virus isolation	PCR/gel electrophoresis	PCR/probe hybridisation
Symptomatic children	292	47 +ve 245 -ve	43 +ve 4 -ve 76 +ve 169 -ve	43 +ve 0 -ve 3 +ve 1 -ve 76 +ve 0 -ve 24 +ve 145 -ve
Asymptomatic children	65	1 +ve 64 -ve	0 +ve 1 -ve 4 +ve 60 -ve	0 +ve 0 -ve 1 +ve 0 -ve 4 +ve 0 -ve 3 +ve 57 -ve
Asymptomatic adults	53	0 +ve 53 -ve	0 +ve 0 -ve 2 +ve 51 -ve	0 +ve 0 -ve 0 +ve 0 -ve 2 +ve 0 -ve 0 +ve 51 -ve

FIG. 4. Summary of results from episode and control samples analyzed by PCR (primer pair OL27-OL26) and gel electrophoresis, PCR (OL27-OL26) and internal-probe hybridization (JWA-1), and virus isolation. +ve, positive; -ve, negative.

Differentiation between HRV and EV by PCR. Sequence data for EV compared with data for HRV indicate an additional ~120-bp insertion at the extreme 3' end of the 5' noncoding region (16). Primer pairs OL68-1-OL26 and OL68-1-OLSJ-1 should therefore yield PCR products ~120 bp longer for EV and HRV. The fragment yielded by primers OL27 and OL26 is the same length for EV and HRV.

Thirty samples that were positive by gel electrophoresis with primer pair OL27-OL26 but were culture negative were subjected to PCR with both primer pairs OL68-1-OL26 and OL68-1-OLSJ-1. Only 8 of 30 samples produced a visible band with primer pair OL68-1-OLSJ-1, while 2 of 30 produced a visible band with primer pair OL68-1-OL26 (all were short and therefore HRV). In view of the poor detection rate, further differentiation between EV and HRV by PCR was not attempted.

DISCUSSION

This is the first study to examine PCR to detect picornavirus genomic sequences in a large number of clinical samples collected in a prospective longitudinal study. It is the only study comparing the performance of several pairs of primers, to confirm all positive results by duplicate testing and internal probe hybridization, and to examine the performance of the assay in samples from both symptomatic and asymptomatic subjects. We have demonstrated (i) a clear superiority in detection rate for one of the primer pairs used (OL27-OL26); (ii) that with internal-probe hybridization, the picornavirus PCR has a detection limit below 10 TCID₅₀/ml; and (iii) that the best PCR assay yields over three times as many positive results as the cell culture virus isolation method.

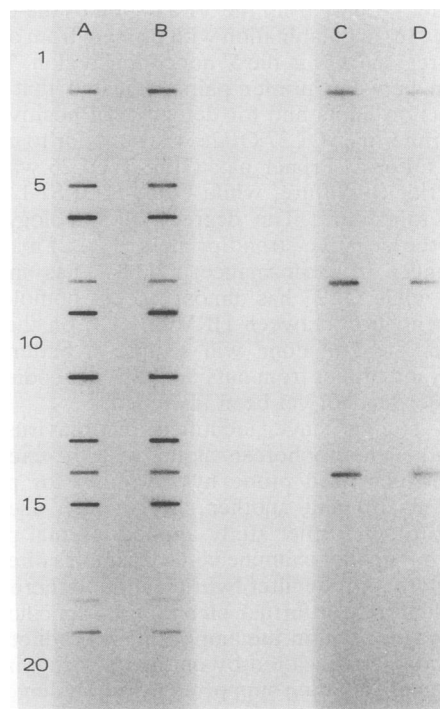


FIG. 5. Autoradiograph of slot blot. PCR products were hybridized with ³²P-labelled internal probe (JWA-1) and exposed for 30 min. Lanes A and B contain duplicate samples from reported episodes; rows 2, 5, 6, 8, 9, 11, 13, 14, 15, 16, 18, and 19 gave positive signals. Lanes C and D contain duplicate samples from asymptomatic children; rows 2, 8, and 14 gave positive signals.

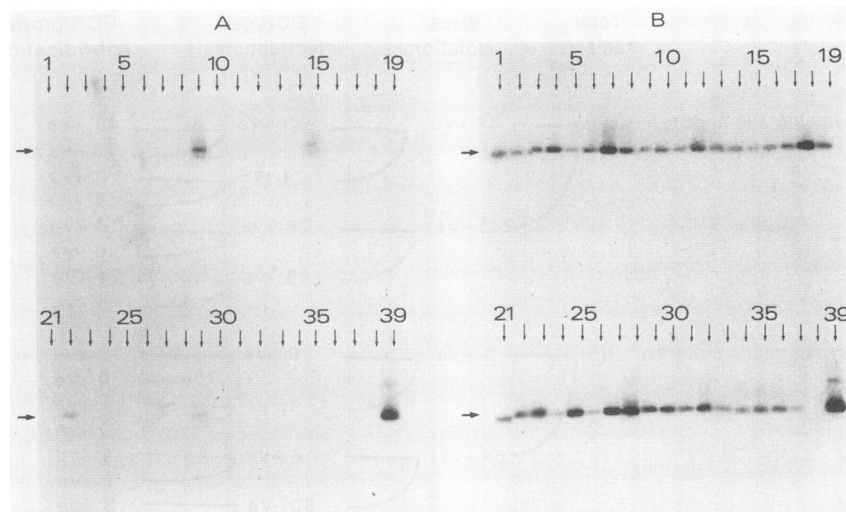


FIG. 6. (A) Southern blot of the first 36 children's control NA samples processed by PCR with primers OL27 and OL26 and gel electrophoresis. Lanes 1 to 19 and 21 to 37 all contain samples, lane 38 contains a negative buffer control, lane 39 contains a positive buffer control, and lanes 20 and 40 contain size markers. The position of the expected product of 380 bp is marked by arrows. Lanes 9, 15, 22, 27, 29, and 36 demonstrate hybridization signals colocated with the bands seen in gel electrophoresis. (B) Southern blot of the first 36 episode NA samples that were positive by PCR with primers OL27 and OL26 and gel electrophoresis but were negative for virus isolation. Lanes 1 to 19 and 21 to 37 all contain samples, lane 38 contains a negative buffer control, and lane 39 contains a positive buffer control. The position of the expected product of 380 bp is marked by arrows. All samples demonstrate hybridization signals colocated with the bands seen in gel electrophoresis.

In undertaking this study our aim was to use a set of primers that would distinguish between HRV and EV on the basis of the length of the 5' noncoding region, using the mixed primer OL68-1 from the VP2 region of the picornavirus genome (16) in combination with primers from one of two conserved regions within the 5' noncoding region. The initial comparison between primer pairs indicated that both for lower detection limits and for detection of positive clinical samples, primer pair OL27-OL26 was superior to either pair containing OL68-1. Primer pair OL68-1-OL26 was considerably inferior to culture, while OL68-1-OLSJ-1 was only equivalent to culture. The degrees of homology demonstrated by the negative-strand primers (Fig. 2) may explain the differences in performance; OL68-1 has incomplete homology, while OL27 has almost perfect homology. Efficient differentiation between HRV and EV on the basis of PCR product length alone will require a better-matched negative-strand primer from outside the 5' noncoding region; such a primer has not yet been identified.

Previous studies have identified picornavirus-positive samples by gel electrophoresis alone, with the exception of one study which used probe hybridization for poliovirus types 1 to 3 (16) and another study which used partial sequencing (6). A further study applied internal probe hybridization but did not examine clinical samples directly (12). We used internal-probe filter hybridization to increase specificity by identifying a further picornavirus-specific oligonucleotide sequence within the amplification product.

Picornavirus identification by culture is recognized to be imperfect, an observation supported by our finding that PCR detected three times as many positive samples as culture. In the absence of a "gold standard," we therefore confirmed picornavirus specificity in all positive samples by internal-probe hybridization and in many cases by Southern blotting as well. The good agreement between culture isolates and positive PCR (with 46 of 47 cultured HRV being positive

with the most sensitive PCR assay and the remaining one being positive with an alternative primer pairing) also provides convincing evidence for the picornavirus specificity of the PCR assay.

It is not known whether partial or complete picornavirus sequences may be carried by healthy subjects. Therefore, since we had identified a large number of positive samples in association with symptomatic episodes, it was important to investigate the existence of a carrier state. Of samples from asymptomatic children, 12% were positive for picornavirus by PCR. This is greater than the positive virus culture rate of 3% for all respiratory viruses in asymptomatic children aged 0 to 12 years reported in previous studies (9, 13). Four percent of samples from asymptomatic adults were positive for picornavirus by PCR, a rate similar to the positive viral culture rate of 3% for all respiratory viruses in asymptomatic adults (10).

The present study confirms that picornavirus PCR achieves a high identification rate (50%) in association with symptomatic episodes. Of the picornaviruses in this study, 55 of the 147 (37%) are known to be HRV on the basis of either acid lability or a short 5' region on PCR, while the remaining 92 are unclassified picornaviruses. Because HRV is known to be the major pathogen of the common cold, it is highly likely that the majority, if not all, of the unclassified picornaviruses identified by PCR are HRV.

In conclusion, we have reported the first use of PCR for picornaviruses combined with a hybridization assay using a radioactive internal probe for a large number of NA samples. The results of this study indicate that with NA samples, this PCR assay achieves at least three times the detection rate of the best available cell culture techniques. Primers that distinguish between HRV and EV failed to identify many of the positive samples, though their use did result in a positive classification in a few instances. Internal-probe hybridization increased the yield of positive results over gel electro-

phoresis alone, as well as confirming picornavirus specificity. With the use of this sensitive and specific technique, it should be possible to define accurately the role played by picornaviruses in both adult and childhood acute respiratory illness. The PCR also has the advantage of speed, allowing accurate diagnosis sufficiently early in the course of the illness to permit practical therapeutic intervention.

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ADDENDUM IN PROOF

In order to further examine the picornavirus specificity of the PCR methodology, we took all clinical samples that were positive by PCR but negative on culture and attempted further isolation in Ohio HeLa cells, using two blind passages after the initial inoculation, which was followed by a final passage if the third passage suggested the presence of CPE. Once again, isolates were confirmed by CPE type and acid stability testing.

A further 29 isolates were identified in this way, bringing the total number of viruses isolated to 85, of which 84 (57% of the 147 identified by PCR) have been shown to be rhinoviruses by either acid lability or production of a short fragment on PCR with either OL 68-1-OL 26 or OL 68-1-OL SJ1. The fact that all 29 viruses further isolated were rhinoviruses rather than enteroviruses adds weight to our suggestion that the vast majority of the 63 remaining unclassified picornaviruses are likely to be rhinoviruses as well. The isolation of a further 29 rhinoviruses in this way also confirms the specificity of the PCR assay and its superiority to culture methods.

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